THE ANTIBACTERIAL STABILITY OF A NEW RADIOPAQUE DOUBLE ANTIBIOTIC PASTE

by

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INTRODUCTION

Development of alternative treatment options for immature teeth with a necrotic pulp apart from traditional apexification procedures, which leave the remaining dentinal walls thin and weak, has been a popular topic of study in recent years [1]. A shift from traditional apexification procedures to regenerative endodontic procedures (REPs) has been observed [1, 2]. The goal of REPs is to prompt the creation of pulp or pulp like tissue to develop into the disinfected root canal space of immature teeth with necrotic pulps . This, in turn, allows for continued root formation in length and in width in those immature teeth [1]. Intracanal medicaments and irrigation solutions are a vital component of the REP protocol because of the importance of achieving an aseptic environment inside the root canal system [3, 4]. The stem cells of the apical papilla must also be introduced into the disinfected canal by penetration with a hand file to induce bleeding and the formation of a clot [5, 6]. This is what encourages the undifferentiated apical stem cells to grow and differentiate into pulp like tissue [7].

A compelling clinical challenge is faced when endodontically treating immature teeth with pulpal necrosis due to their thin radicular walls and short blunderbuss roots. These canals are difficult to obturate and are left with a high risk for fracture and poor crown to root ratios [8, 9]. Apexification is a technique traditionally used to endodontically treat these teeth by placing calcium hydroxide or MTA to stimulate a hard tissue barrier at the apex [10-12]. This procedure creates a barrier, but still leaves the tooth weak with a poor long term prognosis [13]. REPs have addressed these problems by encouraging development in length and thickness of radicular dentin [13-15].

The crucial factors needed for regenerative endodontic success are disinfection, stem cells, scaffolds, and growth factors [16]. The protocol currently recommended by the American Association of Endodontists (AAE) is to disinfect the canal then induce bleeding. This blood from the apical papilla contains mesenchymal stem cells [7]. The clot serves as a scaffold in which growth factors which are derived from platelets or dentin can induce hard tissue formation [17, 18]. Disinfection during REPs has included materials such as sodium hypochlorite, calcium hydroxide, triple antibiotic paste (TAP), and double antibiotic paste (DAP) [19, 20].

Sodium hypochlorite is an effective irrigation solution and has been used for many years in endodontics due to its ability to dissolve tissue and disinfect [19]. The drawback of its use during REPs is that it is cytotoxic to stem cells [20] and has been linked to a decrease in stem cell attachment [21]. Consequently, its use is only recommended at the first visit in lower concentrations. Additionally, its use should be avoided at the second visit, which is when stem cell growth is desirable [22].

Calcium hydroxide is another bactericidal agent that is known for its alkaline nature and antibacterial properties [23-26]. These desirable effects, however, also come with some negatives. Namely, calcium hydroxide has also caused decreased tooth fracture strength when used for more than 30 days [27], superficial collagen degradation [28], and reduced flexural dentin strength [29].

Hoshino et al. formulated triple antibiotic paste (TAP: ciprofloxacin, metronidazole, and minocycline) and showed it can be used as an effective topical antimicrobial treatment on radicular dentin [4, 30]. There were several drawbacks of this medicament which include dentin demineralization, tooth discoloration from

minocycline, and stem cell cytotoxicity when used at higher concentrations [28, 31, 32]. Therefore, its suggested use in REPs is at lower concentrations [22, 33, 34].

Another topical medicament used successfully in REPs is double antibiotic paste (DAP), a mixture of metronidazole and ciprofloxacin [35]. DAP is also cytotoxic at higher concentrations [33]. Ciprofloxacin and metronidazole both prevent the replication of bacterial DNA, each by a different mechanism. Metronidazole works by altering the helical DNA structure and ciprofloxacin inhibits DNA gyrase [36, 37]. DAP is recommended to be used at lower concentrations which are sufficient for it to be antimicrobial while minimizing stem cell cytotoxicity [33].

DAP has been used as a topical antibiotic medicament for several cases of REPs [38, 39]. One study in a case of an immature tooth with a necrotic pulp placed a mixture of metronidazole and ciprofloxacin (DAP) in the canal system and monitored the tooth over a several month period [40]. The thirty-month follow-up radiograph showed evidence of thickened dentinal walls and apical closure [40]. Nevins and Cymerman also used DAP in four clinical cases of regenerative endodontic therapy in which they found reduced sizes of periapical lesions and a significant reduction of symptoms [41]. They also established that the exclusion of minocycline did not negatively affect tissue repair [41].

Additional studies have compared DAP to TAP and calcium hydroxide against common endodontic pathogens [*Enterococcus faecalis*) [38]. These studies found that DAP had antibacterial results equal to TAP [42] without the discolorization [43]. Additional findings include that DAP has antibacterial affects against *E. faecalis* at 0.125,

0.25, 0.5,1, and 10 mg/mL dilutions, but the 0.125 mg/mL dilution is the least cytotoxic to dental pulp stem cells [38].

DAP is not radiopaque, requiring components to be added to it to be used as a commercial material. Radiopaque components are usually added to endodontic materials (cements, pastes, sealers, and obturating materials) to provide radiopacity and determining the exact location of the root canal material in the root canal system. These radiopaque components are usually water-insoluble salts of heavy metals such as barium, zirconium and bismuth. Unlike commercially available calcium hydroxide pastes, all traditional antibiotic mixtures used during REPs lack radiopacity and therefore cannot be visualized on radiographs. Furthermore, all commercially available antibiotic intracanal medicaments, specifically Odontpaste and Ledermix paste, are not radiopaque. Therefore, the use of these antibiotic medicaments are limited in the United States as they are not helpful in detecting lateral and accessory root canals, areas of root resorption or fractures, and the degree of apical development. The incorporation of radiopaque agents into antibiotic medicaments may offer an additional diagnostic aspect to these pastes as well as help the clinicians to recognize the extrusion of antibiotic paste beyond the root canal system. The accuracy of its placement is of great importance during REPs due to the presence of large blunder buss apices of immature teeth with necrotic pulps. However, the addition of these insoluble radiopaque agents may interfere with the antibacterial abilities of the antibiotics and render these antimicrobials inefficient. A recent study here at IUSD introduced a radiopaque DAP with comparable radiodensity to the commercially available calcium hydroxide paste. The proposed radiopaque DAP composed of 30%

insoluble barium sulfate as contrasting agent. However, the antibacterial stability of this radiopaque intracanal medicament is yet to be determined.

In this study, we will investigate the antibacterial stability of a new radiopaque DAP applied to infected dentin specimens immediately after preparation, as well as after 3 and 6 months of storage. To the best of our knowledge, no previous study has explored the antibacterial stability of radiopaque antibiotic pastes to be used for endodontic disinfection. The radiopacity of this DAP may prove to be more clinically useful for REPs for clinicians to be able to visualize the placement of the medicament radiographically.

OBJECTIVE

The aim of the current study will be to evaluate the antibacterial stability (shelf life) of two concentrations of the new radiopaque DAP for up to 6 months.

HYPOTHESES

Null: Dentin treated with radiopaque DAP will not demonstrate significant antibacterial effects regardless of the storage time of the pastes.

Alternative: Dentin treated with radiopaque DAP will demonstrate significant antibacterial effects regardless of the storage time of the pastes.

REVIEW OF LITERATURE

STORY OF ENDODONTICS

Remarkable advancements have been made in dentistry since the beginning of the profession. Some of the earliest recorded history of dentistry dates back to 5000 BC, with ancient Sumerian texts which discussed the presence of worms inside teeth being the cause of toothaches. This was termed the “tooth worm” theory. In 1684, Anton Von Leeuenhoek disproved this theory by identifying microorganisms from tooth samples under a microscope. The first book in English devoted solely to dentistry was published in 1687 by Charles Allen. The book did not discuss endodontics, but proposed options for treating problematic teeth by transplantation. This method involved removing the problematic tooth and stumps followed by replacement with intact ones [44].

As early as the 1700s, treatments were attempted to search for relief from pulpal and periapical disease while maintaining one’s natural tooth. These experimental treatments involved incomplete chemomechanical treatment of the pulp and obturation of the pulp chamber. Pierre Fauchard was the first to publicly present these ideas [44, 45]. He later wrote a book titled *The Surgeon Dentist*, where he explained many dental and endodontic concepts including endodontic access preparation of an abscessed pulp chamber in order to allow drainage. Fauchard was also the first to propose obturation in endodontics with the suggestion of obturating the pulp chamber and canals using lead foil. In 1756, Philip Pfaff discussed pulp capping with the use of gold or lead [46]. A description of endodontic therapy by using a method of intentional extraction then replantation was described by Bourdet in 1756 [44].

Robert Woofendale was the first to perform endodontics in the US in the 18th century. His method was to cauterize the pulp with hot instruments in order to relieve pulpal pain in addition to using oil of cloves, cinnamon, opium, turpentine, and camphor [47, 48]. Also in the 1800s, Frederick Hirsch proposed percussion testing as a method of diagnosing periapical disease. He then described pulpal treatment by insertion of a heated probe into the pulp [45].

The dental community became interested in the study of the pulp and periradicular physiology from 1800-1850. The concept of pulp vitality and the ability to retain teeth with non-vital pulps was introduced by J.B. Gariot in 1805 [49]. This is the time that pulpal anesthesia and new instruments for canal debridement were introduced [44]. Additionally, the significance of pulp vitality and its associated treatments was recognized. Edward Hudson became the first to place gold foil in the canal space in 1809 [50]. Charles Bew later introduced the idea of pulpal circulation, and the thought that blood flows into the tooth through the apical foramen and exits through the periodontal membrane [49]. In 1826, Leonard Koecker wrote a book entitled, *Principles of Dental Surgery*, where he opposed the idea that a non-vital tooth could be maintained and stated that removing the pulp would cause the tooth to die, causing the body to recognize it as a foreign material. He proceeded to encourage a pulp capping procedure to prevent necrosis which was similar to the ideas of Pfaff [48, 49, 51]. The “vitalistic theory” came to fruition in the 1829 by SS Fitch in his book *System of Dental Surgery.* The idea he proposed was that similar to hollow bone, the whole tooth is vital, with the tooth crown being supplied by pulpal circulation and the root being supplied by both pulpal circulation and periodontal ligament. This theory resulted in the procedure of

decoronation, leaving the root in the socket and restoring the tooth with a crown. The theory originating from the opposite school of thought was termed the “nonvitalistic theory,” supporting the belief that enamel and dentin lack circulation, sensibility, and self-repair potential. Therefore, they believed pulp removal would not affect the tooth [49].

The next concept to come about in the history of endodontic treatment was the use of intracanal medicaments. Painless pulpal debridement was introduced by Shearjashub Spooner in 1836. He proposed devitalizing the pulp prior to removal by using arsenic trioxide. This technique became very popular because it was effective in reducing the pain associated with endodontic procedures [45, 52, 53]. One year later, Jacob and Joseph Linderer described the use of narcotic oil to anesthetize the pulp [46]. The first endodontic broach was invented by Edwin Maynard in 1938 [54]. The first complete written description of root canal therapy was described by Baker in 1839. He described the pulpal debridement, cleaning, and filling of the canals with gold foil [45].

Diagnostic tests, new instruments, disinfectant, obturation materials, prognostic factors, and surgical endodontics came about in the second half of the nineteenth century. Wood plugs soaked in creosote were commonly used as a method to fill canals in 1850 [52]. This was the same time that Codman exclaimed that the aim of pulp capping was to stimulate growth of secondary dentin over the pulp [49]. The next year, Hullihen proposed the methods for the first endodontic surgical procedure, including flap reflection, osteotomy, and tooth trephination to cause the pulp to heme and in turn reduce pain [48]. In 1857, prognostic factors for pulp capping were explained by Thomas Rogers. It was only a year after this that Jonathan Taft proclaimed that reparative dentin

was more resistant to decay [46]. Rubber dam isolation was introduced by Sanford Barnum in 1864 [52, 53]. Dr. G. A. Bowman made a huge advancement in 1867 by introducing the modern endodontic obturation material of choice [53]. This was the same time that Clark Dubuque proposed the use of hot gutta-percha as a method of obturation [44]. The method of using electric current for pulp testing was introduced by Migitot in 1867 [55]. In 1870, G.V. Black advocated using zinc oxychloride as a pulp capping material [45]. The idea that pathogenic microorganisms are the cause of pulpal disease came about in 1878 by G.O. Rodgers.

A new theory finally began to replace the vitalism theory in 1879. This was coined the “septic theory.” The thought of the septic theory was that the etiology of pulpal disease was infected teeth so this shifted the focus of endodontic treatment to disinfection [49]. This new septic theory was what lead to the belief that it a necessary aspect of endodontic treatment is an aseptic environment so clinicians strived to eliminate the pathogenic organisms [44]. 1882 was also a big year in endodontic advancements as Arthur Underwood suggested that using antiseptic agents in the pulp space would eliminate pathogens [44]. In the end of the nineteenth century, chloropercha was invented by Dr. Bowman. Chloropercha is a combination of gutta percha and chloroform, and this was used along with gutta percha cones for the purpose of obturation [52].

Groundbreaking advances occurred in the early 1900s for dentistry and endodontics. Among these were the introduction of local anesthetics, the focal infection theory was proposed and refuted, and the concept and importance of canal length and master apical size was proposed. Procaine [Novocaine) was invented by Einhorn in 1905 as a replacement to cocaine. Dental nerve blocks were also proposed and utilized around

this time [53, 54]. The dental x-ray unit was invented in 1913 and became available on the commercial market in 1919 after the Coolidge tube was invented. The Coolidge tube allowed a more focused x-ray beam [56]. The study of endodontic disease was forever changed after this invention because it allowed us to visualize the periapical radiolucencies associated with pulpal and periapical disease [57]. The use of dental radiographs also brought about new concepts such as that of G.V. Black describing radiographic working length and the concept of apical gauging the root canal system [56, 58]. This all led to our enhanced understanding of root canal anatomy and ability to better clean, shape, and seal the root canal system.

The early 20th century was a trying time for endodontics as the focal infection theory was introduced and placed endodontic practice under scrutiny. The focal infection theory proclaimed that the spread of microorganisms from a tooth [focus of infection) could be spread throughout the body and exacerbate or cause various diseases [59, 60]. A lecture given by William Hunter entitled, “The Role of Sepsis and Antisepsis in Medicine,” sparked the rise of this theory. The lecture was published in Lancet in 1911 [60, 61]. In his lecture, Dr. Hunter stated “gold fillings, gold caps, gold bridges, gold crowns, fixed dentures, built in, on and around diseased teeth, form a veritable mausoleum of gold over a mass of sepsis to which there is no parallel in the whole realm of medicine or surgery.” [59]. This led to the trend of physicians recommending extraction of teeth that are endodontically treated or non-vital, with some physicians going so far as to recommend extraction of all teeth to prevent infection [60, 62]. Finally, in the 1930s and 1940s, the discovery was made that there was not a causative relationship between dental infection and systemic diseases. In 1937, Logan proposed the

distinction between the presence of bacteria and infection [47]. It was around this same time that Tunicliff and Hammond discovered microorganisms in healthy pulps [56, 58]. Also around this same time, Burket documented 200 unresolved arthritis cases although infectious foci had been removed. This disproved that there was a causative relationship between the infectious foci and arthritis, suggesting that it was a serendipitous finding [56]. Due to these findings, the focal infection theory of endodontics began to lose credibility, and initiated the beginning of the "scientific era" [61].

The use of antibiotics became popular in the middle of the 1900s. In the 1940s, Drs. Adams and Grossman introduced antibiotics as an adjunct to endodontic treatment [61]. Grossman first recommended using non-aqueous penicillin as an intracanal medicament and later recommended the use of paper points impregnated with antibiotic inside the canal space [52]. This sparked an interest in chemotherapeutic root canal treatment, but it was discovered that antibiotics alone would not sterilize the root canal space. From this, the concept of mechano-chemical preparation came about. This involved the mechanical preparation of a space which would allow the chemicals to penetrate and disinfect the canal space [63].

The American Association of Endodontists (AAE) was founded in 1943 in Chicago, IL, marking the establishment of organized endodontics. This, in turn, lead to the establishment of endodontics as a specialty in 1949, which then became recognized by the American Dental Association in 1963 after establishment of the American Board of Endodontics (ABE) in 1956 [64].

From the latter part of the 1900s through the start of the 21st century, many new developments came about in the field of endodontics. These included the introduction of

nickel titanium rotary files, enhanced magnification with microscopes, microsurgical instruments, cone beam computed tomography, and materials that are more biocompatible such as mineral trioxide aggregate and bioceramic calcium silicate based materials. These modern inventions are all examples that allow for more accurate endodontic diagnosis and treatment [65-70]. This is also the era in which the field of regenerative endodontics began to gain popularity. The first regenerative endodontics conference was held in 2006 at Nova Southeastern University [71]. Between 2001 and 2010, the AAE dedicated one-half million dollars to 29 regenerative projects [72]. In 2012, the ADA added a new code (D3354) for pulpal regeneration [73]. The topic of regeneration has, in fact, gained so much attention that the *Journal of Endodontics* now dedicates a section in each edition towards new research in regenerative endodontics.

THEORY OF ENDODONTICS

In 1965 Kakehashi, Stanley and Fitzgerald published a study that formed the basis of modern endodontic theories and practices. Their experiment demonstrated that the pulps of germ-free rats remained vital although they were open and exposed. They showed that pulpal necrosis was not caused by occlusal trauma, food impaction, or exposure to the oral cavity. These germ-free rat pulps were also able to heal and maintain health. They also placed conventional [non germ-free) rats under the same conditions and they showed changes of inflammation and pulpal necrosis [74]. In 1981, Moller et. al. also performed a crucial study in the development of current endodontic theories and practices. Their study showed that infected pulp tissue, and not necrotic tissue alone, caused periapical inflammation in monkeys [75]. These two studies proved the crucial role that microorganisms play in the development of periapical pathosis.

The overall goal in the treatment of endodontic infections is to maximally reduce the microbial load in order to reduce and eliminate pulpal and periapical pathosis, while also restoring function of the tooth [76-79]. If the microbial load is not reduced sufficiently, apical periodontitis could result. The definition of apical periodontitis is destruction of the periodontium with or without producing symptoms [80]. There is sufficient evidence to show that there is a direct correlation between endodontic success and the reduction of microorganisms [81].

Stewart, Grossman, and Schilder described in the classic literature the most important endodontic practices for optimal endodontic outcomes [76, 77, 82, 83]. In 1955 Stewart was the author who described the separation of endodontic therapy into three distinct phases. These included chemomechanical preparation, microbial control, and obturation [82]. Stewart's work gained the most attention for the chemomechanical preparation phase. It was Grossman who later confirmed the findings of Stewart that, indeed, chemomechanical preparation is necessary in order to maximally reduce pathogenic bacteria and their toxins. Grossman took these concepts even further and identified these 13 major principles of effective root canal therapy:

1. Aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic.
6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.

8. A negative culture should be obtained before obturation of the root canal.

9. A hermetic seal of the root canal system should be obtained.

10. Obturation material should not be irritating to the periapical tissues.

11. If an acute alveolar abscess is present, proper drainage must be established.

12. Injections into infectious areas should be avoided.

13. Apical surgery may be required to promote healing of the pulpless tooth.

It wasn't until 1967 that Herbert Schilder introduced obturation of the root canal in three dimensions [76]. He also emphasized that the main objective of root canal treatment is to eliminate diseased tissue and tissue of the inflamed or infected pulp. In conclusion, it was these three authors who pioneered the identification of the three phases of root canal therapy: instrumentation, irrigation, and obturation.

INSTRUMENTATION

Instrumentation is defined as the enlargement of the canal space to the extent of the apex in order to permit irrigation solutions to access the entire canal space [83-85]. This is considered the first phase of endodontic treatment. It is of critical importance to maintain the original shape of the canal and not to force instruments beyond the apical constriction as to prevent damage to the periodontium [83, 86]. It has been shown in the literature that instrumentation does reduce the bacterial load of the root canal drastically; however, 35 percent to 53 percent of the canal walls are untouched [87-89]. Furthermore, bacteria are not only inside the canal, but also penetrating into the dentinal tubules, lateral and accessory canals. This is proof that mechanical instrumentation alone will not sufficiently reduce the bacterial load and therefore irrigation is needed in conjunction with instrumentation [90, 91].

IRRIGATION

Continuous irrigation with antiseptic solutions during endodontic therapy is of utmost importance. The irrigants also must remain in the canal for an adequate amount of time [92]. Sodium hypochlorite (NaOCl) solution is recommended as the primary irrigating solution. This is due to its ability to dissolve organic tissues and its efficacy in broad spectrum antimicrobial activity [93]. NaOCl has a high pH of 11. This allows the hypochlorous acid to have a strong antimicrobial activity. The hypochlorous acid disrupts oxidative phosphorylation, DNA synthesis, and membrane activities [94-96]. Temperature, exposure time, and concentration all influence the efficacy of NaOCl. These factors are also directly proportional to the degree of tissue dissolution and dentinal tubule penetration [92, 97, 98]. NaOCl does have some drawbacks, the main one being its inability to dissolve the smear layer which may also block its penetration into the dentinal tubules [99, 100]. This problem was remedied by the implementation of another solution, Ethylenediamene Tetra-Acetic Acid (EDTA) [99]. EDTA sufficiently removes the smear layer of the root canal system with one minute of irrigation [101]. With the removal of the smear layer, there is improved penetration of the NaOCl and an improved fluid-tight seal following root canal obturation [102, 103]. Two drawbacks of NaOCl is its lack of substantivity and ineffectiveness against endotoxins [104-106]. Another supplemental irrigation solution that is frequently used in addition to NaOCl is Chlorhexidine gluconate (CHX). CHX has been shown to have broad-spectrum antimicrobial activity in addition to substantive antimicrobial effects for up to a period of 12 weeks [107, 108]. CHX is a cation and its antimicrobial effects come from electrostatically binding to bacterial cell walls and disrupting them as well as PCA [109,

110]. A negative side of CHX is the formation of a harmful precipitate when mixed with NaOCl. Recent studies have found this precipitate to be parachlorophenylurea (PCU) and parachlorophenylguanidyl-1,6-diguanidyl-hexane [PCGH) [111, 112]. When using CHX and NaOCl together, the canal should be flushed between in order to prevent formation of the precipitate [112].

OBTURATION

The final step of endodontic therapy aims to create a hermetic seal of the root canal. This process is termed obturation. It is very important for the obturation materials to be biocompatible so as to prevent irritation of the periapical tissues [83]. According to one systematic review, obturation will have a high success rate when termination of the material is 0 to 1 mm from the radiographic apex [113]. Later, the results of this systematic review were confirmed with an outcome study showing that the two most significant prognostic factors for root canal success were length and density [114]. Furthermore, endodontic sealer is imperative in order to ensure the best seal of the root canal system [113].

A definitive restoration following root canal treatment is of high importance once the root canal system has been cleaned and obturated. This is in order to prevent crown down bacterial leakage [115]. In order to have a successful root canal outcome, many steps are taken to reduce the microbial load inside the tooth as much as possible.

MICROORGANISMS

The microorganisms involved in endodontic infections are typically classified into primary and secondary infectious bacteria. Bacteria present in these infections exist in

communities known as bacterial biofilms. A bacterial biofilm is a dynamic organization of complex biologic systems that provides the bacteria with many positive influences for their survival and virulence [115].

Primary endodontic infections are composed of mostly gram-negative anaerobic rods [76, 116]. In 2014, Nagata showed infected immature teeth to have similar microbial composition as primary endodontic infections of permanent teeth, averaging 2.13 species per root canal [117]. The most prevalent species found in infected root canals of immature teeth is *Actinomyces naeslundii* [117]. Additionally, *A. naeslundii* is found within the normal microbial flora of plaque, caries and primary endodontic infections. It is capable of binding to epithelial cells, salivary proteins, and the surfaces of teeth [118, 119]. The virulence factors of these bacteria activate the innate immune system and lead to a release of cytokines, which in turn causes inflammation [120-123].

F. nucleatum is also a primary endodontic pathogen to note. These bacteria are gram-negative and non-spore forming. In the shape of a fusiform rod, *F. nucleatum* can be found often in patients with periodontal and periapical pathology [123, 124]. *F. nucleatum* plays a key role as a middle colonizer in biofilm formation as it enables several other bacteria to attach around it.

Another notable bacterium that is a gram negative, obligate anaerobe, black-pigmented bacterium is *P. gingivalis.* This pathogen has some major virulence factors including fimbriae, lipopolysaccharide, capsule, and lipoproteins. *P. gingivalis* has been identified in as many as 50 percent of primary endodontic infections. Its presence has also been identified in the tongue, tonsils, and gingival sulcus. For complete irradication

of *P. gingivalis* from the canal system, one minute of irrigation with 1.0-percent NaOCl is adequate [128].

When a microbe that is not present during initial treatment but later gains access through the canal system during root canal treatment, this is termed a secondary endodontic infection [115, 126]. Secondary endodontic infections typically present with a mixed flora type composed in large part of gram-positive facultative cocci shaped cells and an average of 1.3 species per root canal [116, 129]

E. faecalis is one of the most common bacteria encountered in both secondary and persistent endodontic infections. It is a facultative anaerobe and stains gram-positive [130]. *E. faecalis* is able to remain prevalent in persistent infections due to its ability to resist the high pH of $Ca(OH)_2$ by "hiding out" in the depths of the dentinal tubules [115, 126, 129, 131]. It has its own set of effective virulence factors that allow it to form a biofilm making it more resistant to antibodies, phagocytosis, and antimicrobials than other microbial organisms that do not form a biofilm. The main virulence factors of *E. faecalis* include lytic enzymes, aggregation substance, cytolysis, pheromones, and lipoteichoic acid [132, 133]. *E. faecalis* organisms contribute to the challenging nature of endodontic therapy, especially in the treatment of immature permanent teeth with necrotic pulps.

IMMATURE TEETH WITH NECROTIC PULPS

Immature permanent teeth with pulpal necrosis present a clinical challenge to the endodontic practitioner. These teeth do not show the high success rates of endodontically treated permanent teeth [134]. Their compromised prognosis is due to their underdeveloped, thin, short roots that increase the risk for endodontic or restorative

failure [8, 9]. Additionally, obturation of immature necrotic teeth is difficult due to the high risk of overextending the filling material beyond the apex [43]. Because of these difficulties, different treatment protocols have been implemented for the treatment of the immature tooth with a necrotic pulp.

APEXIFICATION

Apexification is a procedure that was embarked upon in the 1960s for the management of treating immature teeth with necrotic pulps and an open apex. This was achieved by placing calcium hydroxide as an intracanal medicament for a long period of time [11]. The long term calcium hydroxide treatment creates a barrier termed an apical bridge. The apical bridge consists of osteoid or cementoid tissue and serves as a barrier for the operator to condense obturation material against [11, 135].

An access preparation is made through the tooth to gain access to the canal space using rubber dam isolation. A working length is then determined followed by minimal instrumentation of the canal. The disinfection of these large canals is achieved mostly through irrigation. The next step is the placement of calcium hydroxide into the canal space and leaving it for several months. Typically, the medicament is left in the canal for a period of 9 to 24 months with the patient being recalled at 3 months intervals to examine progress of apical barrier formation. When an adequate apical barrier has been formed, the canal is obturated with gutta percha or MTA followed by placement of a definitive restoration [136].

Although apexification creates a barrier for obturation of the large open apex, it also has some limitations. These include failure to increase thickness and length of root canal walls leaving teeth very prone to root fracture [6, 137-139]. The incidence of root

fractures with these teeth can be as high as 77%, depending on the stage of root development [8].

A modern modification of endodontic apexification procedures involves using an artificial apical barrier technique. This is an alternative to using long-term calcium hydroxide. This technique uses the same protocol for access and disinfection as traditional apexification procedures. The difference is calcium hydroxide is placed for a period of just 1-2 weeks in order to disinfect the root canal system. When the tooth is asymptomatic, the calcium hydroxide is rinsed from the canal, dried, and an apical collagen barrier is placed followed by condensation of 4 mm of MTA at the apex [140]. The tooth is then filled with gutta percha to the orifice and a proper coronal restoration placed. This technique has many benefits including reduced treatment time and the ability to place a coronal restoration sooner, which leads to reduction in crown down leakage and cervical root fracture. This procedure has a very high success rate of 85-93.5% [141, 142].

These high success rates show that modern apexification techniques have increased the prognosis of immature teeth with necrotic pulps, but their long-term prognosis is still compromised due to the remaining root walls being short and thin. Thankfully, the advent of regenerative endodontic therapy has begun to provide a more favorable alternative and better outcome for the endodontic treatment of immature teeth with necrotic pulps.

REGENERATIVE ENDODONTICS

Regeneration, tissue engineering, and stem cell research has begun to get a lot of attention in the fields of dentistry and medicine. The definition of regeneration is the

development of biological substitutes that restore, maintain, replace or improve the function of tissues [143]. There is an important difference between healing by regeneration and healing by repair. Repair healing means the new tissue is not identical to the original tissue or there has been loss of structure or function. Alternatively, when healing by regeneration occurs, there is new tissue that is identical to the original tissue it is replacing. Also, the original structure and function are restored [144]. Tissue regeneration requires three main factors: stem cells, scaffolds, and growth factors [143]. Regenerative endodontics is a procedure which induces tissue regeneration. This is achieved by disinfection of the root canal system prior to induction of bleeding from the apical papilla, where stem cells are located [7, 144, 145]. The goal of regenerative endodontic procedures is to replace the damaged structures of the pulp-dentin complex including the dentin and root structures [146].

TERMINOLOGY

Different terms have been used to describe regenerative procedures. Initially, it was termed “revascularization.” This is defined as restoring the vascularity. This terminology is not appropriate because revascularization occurs in all healing whether it is by regeneration or repair [147]. Another faulty aspect of this terminology is that the newly generated tissue inside the root canal is not always vascular in nature. This is why the term “revitalization” was then proposed as an alternative term to “revascularization.” [22]. The problem with the term “revitalization” was that it became commonly misinterpreted as implying re-innervation. The AAE has since re-evaluated the goal of regeneration and, after careful consideration, recently began using the term “regenerative

endodontic procedures." (REPs) [148]. This more accurately describes the ideal goal of regeneration than the terms "revascularization" or "revitalization."

HISTORY OF REGENERATIVE PROCEDURES

Regenerative endodontics was conceptualized in 1961 by Nygaard-Østby. His initial test was to see if healing is promoted with the presence of a blood clot inside the root canal system. His experiment involved seventeen patients with either necrotic or vital pulps. Each tooth had root canal therapy followed by enlargement of the apical foramen and an intracanal medicament was placed. He then initiated intracanal bleeding through stimulation of the apical tissue. Restorations were placed in the teeth and followed up for a time period of 17 days to 3.5 years. These teeth were later extracted and examined histologically. The findings were that all teeth were asymptomatic without inflammation or pathosis. There were even some cases that portrayed evidence of apical closure [149]. Histologically, in-growth of some connective tissue was seen in some of the cases of previously vital canals. This connective tissue was not the same as pulp tissue and was lacking odontoblast cells [22].

The first report on a study involving the use of poly-antibiotic pastes to disinfect necrotic teeth and encourage root development was published in 1966 [22]. In 1974, infected mature and immature teeth were treated by Myers. In his report, he described disinfection of the canals with 5.25% NaCl followed by apical enlargement of the foramen and bleeding evocation. After 24 weeks, tissue growth was seen. But, there was also incidence of root resorption and periapical inflammation. This could possibly be explained by coronal leakage or incomplete disinfection of the canal spaces. One important discovery of this study was that immature teeth showed a more favorable

response to treatment than mature teeth. The immature teeth showed evidence of continued root growth as well as a larger amount of connective tissue within the canal space [150]. It was only two years later that Nevins treated immature teeth in monkeys which were pulpless. His treatment included biomechanical debridement and placement of collagen-calcium phosphate gel for a period of 12 weeks. These teeth were examined histologically and he found some different forms of connective tissue which included "cementum, bone, and reparative dentin' [151].

RECENT DISCOVERIES

Due to recent discoveries, we now have more knowledge on REPs. Iwaya was the first to publish a case report of contemporary regenerative endodontic treatment in 2001. He used a double antibiotic paste (DAP) consisting of metronidazole and ciprofloxacin for the treatment of an immature tooth with necrotic pulp and a periapical lesion. Instrumentation was minimal in order to conserve vital tissue in the apical portion which could potentially aid in revascularization. Therefore, canal disinfection was achieved by irrigation with solutions of 3% H_2O_2 and 5% NaOCl followed by intracanal application of DAP. He then placed a layer of calcium hydroxide approximating the apical tissue and restored the access cavity with a definitive resin restoration. The result was radiographic evidence of continued root growth and apical closure at the 30-month follow up [152].

Three years later, Banchs and Trope published a case report of a mandibular premolar with an immature root and necrotic pulp which was treated using triple antibiotic paste (TAP), consisting of ciprofloxacin, minocycline, and metronidazole. They detailed a protocol for revascularization of immature teeth with necrotic pulps

which was based on the healing of avulsed permanent teeth with immature roots [153]. Their hypothesis was formed with the idea that if a similar environment was created for the immature tooth with necrotic pulp, revascularization could possibly occur. The protocol developed started with chemical canal disinfection with 5.25% NaOCl, Peridex, TAP for 4 weeks, and then 5.25% NaOCl again. The next step was inducing apical bleeding with an explorer tip until blood filled the canal to the CEJ. This was left to clot for 15 minutes. The next step involved placing a final seal with MTA. At a follow up time of 2 years, there was complete resolution of symptoms, continued root growth, and a positive response to cold testing. Thereafter, this protocol has been repeated by several practitioners and resulted in many successful regeneration case reports [154-157].

The three requirements for REPs were identified by Nakashima et al. in 2005. These include stem cells, a scaffold, and growth factors [158]. It was in 2001 that Lovelace was able to quantify the mesenchymal stem cells in blood of the apical area. He concluded that the number of stem cells in this area was 600-fold greater than the levels found in systemic blood [7]. The scaffold for growth of new vital tissue into the canal space is the blood clot, as explained by Banchs and Trope [153]. In 2009 Bose et al. quantified root development after REPs and reported a 25.1-percent increase in width and 14.7-percent increase in length [15]. In 2012 Jeeruphan et al. found a 28.2-percent increase in width and 14.9-percent increase in length [13]. The conclusions of both of the above mentioned studies confirmed that tooth development is significantly more with REPs than for MTA or $Ca(OH)_2$ apexification. In 2014 Kahler et. al. did a report on a case series of 16 REPs and concluded that there was resolution of apical pathology in 90.3% with complete apical closure in 19.4% at 18 months [159].

A variety of findings have been reported on what type of tissue is found in REPs. Yamauchi et. al. did a study on dogs in 2011 and found two types of new tissue associated with immature treated dog teeth. These included bony islands (BI) and dentin-associated mineralized tissue (DAMT). The bony islands were highly cellular and found within the canal lumen, whereas the DAMT did not contain vasculature, was less cellular, and located against the dentinal wall. In 2012, Wang et. al found three tissue types in treated immature dog teeth. These were intracanal bone (IB), intracanal cementum (IC), and other connective tissue. Also in 2012, Shimizu et. al. found cells resembling odontoblasts and loose pulp-like connective tissue. Later in 2013, Martin et. al. did an in vivo REP case and reported fibrous connective tissue and mineralized tissue, but no pulp-like tissue or odontoblast-like cells [152, 153, 160-163].

The above mentioned studies all had some common features which led to the early successes reported for REPs. These common features were young patients with open apices, minimal instrumentation was performed, calcium hydroxide or TAP was used as interappointment medicament, and formation of a blood clot provided a scaffold [164]. The documentation of these cases formed a foundation for REPs and became the gateway into the development of the modern REP protocols.

INDICATIONS AND OUTCOMES FOR REPs

REPs are primarily intended for the treatment of immature teeth with necrotic pulps of adolescents due to trauma studies suggesting that apical diameters greater than or equal to 1 mm have a higher possibility to revascularize [165]. However, Laureys et. al. found that revascularization was permitted in dog teeth with an apical foramen as small as 0.32 mm, suggesting the size of the apical foramen may not be as crucial as was

once believed [166]. The AAE has set three goals for defining and measing the outcome success for REPs: 1) periradicular healing and eliminating symptoms completely, 2) continued root growth, and 3) positive response to sensibility tests [73]. The three pillars of REPs have been formed by many studies: disinfection, stem cells and growth factors, and a scaffold.

DISINFECTION AND IRRIGATION

Through their famous experiment on germ-free rats, Kakehashi, Stanley and Fitzgerald formed the foundation for all endodontic treatments [74]. Like any other form of endodontic treatment, REPs require disinfection of the root canal system. This was proved by an experiment performed by Thibodeau et. al. in 2007. This experiment involved performing REPs on immature teeth with necrotic pulps of dogs. They then confirmed histologically the presence of vital tissue only when teeth were disinfected first [167]. Most commonly, the disinfection regimen used begins with irrigation with NaOCl then placing an intracanal medicament with either calcium hydroxdide or antibiotic pastes [168].

Sodium hypochlorite (NaOCl) continues to be the most commonly used intracanal irrigant used in the practice of endodontics. It was first introduced by Coolidge in the year 1919 [54, 164]. It is a very desirable chemical irrigant for root canal treatment due to its extreme antibacterial activity and ability to dissolve organic tissue [119, 170, 171]. Concentrations of NaOCl used for endodontic therapy range from 0.5%-8%. Using a lower concentration, such as 1.5%, serves to dissolve necrotic tissue and minimize vital tissue destruction [172]. Even though NaOCl is a potent antimicrobial, it also has some disadvantages when used for REPs. It is known to have cytotoxicity on stem cells which

is concentration-dependent, and can impact stem cell attachment [173, 174]. There has also been a correlation found between the use of NaOCl at concentrations of 3% and 5% and a reduction in flexural strength of dentin. These are all reasons that the use of NaOCl in a concentration of 1.5% is recommended for REPs during the disinfection phase. Furthermore, its use is completely avoided during the second phase of REPs when bleeding of the apical papilla is induced [73].

Calcium hydroxide ($Ca(OH)_2$) is an intracanal medicament introduced in 1920 by Herman. $Ca(OH)_2$ is an effective antimicrobial due to its ability to inactivate and detoxify the endotoxins of lipopolysaccharide (LPS). LPS endotoxin is responsible for the inflammation of the periapical tissues, so its inactivation is critical to aid in the process of periapical healing [115, 175, 176]. Additionally, calcium hydroxide has an alkaline pH and exerts its effects by releasing hydroxyl ions. These hydroxyl ions create free radicals which inhibit replication of DNA and the bacterial cell activity through direct contact with bacteria. Additionally, the alkaline pH (12.5) of calcium hydroxide denatures enzymes and retards cellular metabolism of bacteria [177]. There was an in vitro study performed which showed that 24 hours of calcium hydroxide treatment led to complete killing of enterococci [115, 178, 179].

Calcium hydroxide is also approved for use in regenerative endodontic procedures as this has been successful in case series reports [13, 180]. Additionally, calcium hydroxide is conducive to the survival of stem cells of the apical papilla (SCAP), and actually causes an increase in the survival of SCAPs at 1 mg/mL [33].

Calcium hydroxide does, however, have some drawbacks. The use of pure calcium hydroxide has a negative effect on the OPG/RANKL ratio, inhibiting hard tissue

formation [181]. Because of this, calcium hydroxide is typically compounded with saline or sterile water before being administered into the root canal system. In a study by Andreasen et. al., four week application of calcium hydroxide in REP cases decreased the tooth fracture strength [27]. In a similar study by Yassen, three month application of calcium hydroxide on extracted teeth caused an increase in microhardness and a reduced fracture resistance [182]. Additionally, superficial collagen degradation was reported after a one to four week application [183].

The purpose of endodontic therapy on an immature tooth, historically, is to resolve apical inflammation. One of the most important steps in achieving resolution of apical inflammation is eliminating bacterial load inside the canal system. Since infections of endodontic origin are polymicrobial in nature, single antibiotics are not able to eliminate all the bacteria, especially when biofilm layers are present [184]. This is the reason Hoshino et al reported the use of TAP, a combination of ciprofloxacin, metronidazole and minocycline [4, 185]. Each of these components has a different function in the killing of bacteria. Ciprofloxacin and metronidazole both prevent bacterial DNA synthesis, whereas minocycline targets protein synthesis by binding to the 30s ribosomal subunit. The efficacy of TAP may be attributed to the broad spectrum bactericidal effect of metronidazole [37]. Studies performed in vitro have shown that 0.3 mg/mL of TAP effectively eliminates bacteria cultivated from endodontic lesions [186].

Like other materials used for disinfection, TAP also has some limitations and drawbacks. Some of these are discoloration, demineralization, and cytotoxicity. It is important to note that minocycline causes dentin discoloration [160, 187]. This is because minocycline acts as a chelator which binds calcium ions, forming an insoluble complex

that incorporates into the tooth matrix [188]. Minocycline's chelating effect along with its acidic pH of 2.9, is what causes demineralization [183]. Most concerning is the stem cell cytotoxicity of TAP. Initially, concentrations as high as 1000 mg/mL were used. It has been shown that concentrations as high as 1, 10, and 100 mg/mL have been detrimental for the survival of SCAP [33]. Ruparel found 50 percent of cells died when exposed directly to 1 mg/mL TAP. Due to this, lower concentrations of TAP have been recommended [73]. An additional drawback of TAP is that it is not readily available and must be made by a compounding pharmacy. Another medicament that has been shown to have severe cytotoxicity to SCAP is 2% chlorhexidine. Because of this, chlorhexidine is completely contraindicated in REPs [114].

The next intracanal medicament to discuss is ethylenediaminetetraacetic acid (EDTA). Described by Ferdinand Munz in 1935, it is a chelator which is used to remove the smear layer, which is the inorganic portion of the dentin [100]. The smear layer is composed of the debris created by mechanical instrumentation along with bacteria and byproducts. The significance of the smear layer is that it clogs dentinal tubules in the root canal system. EDTA has the ability to sequester metal ions such as calcium and iron. Additionally, it can kill bacteria through direct exposure to surface proteins for an extended time period [115].

In its use for REPs, EDTA improves the potential for regeneration in several ways. Use of 17% EDTA removes the smear layer and exposes dentinal tubules. This is very important for creating an environment for regeneration because these exposed dentinal tubules have been shown to cause the release of growth factors from the dentin [189-191]. EDTA has also been shown to increase the surface roughness of dentin, which

can increase the attachment of growth factors to dentin [192]. Dental pulp stem cells have shown an increased attraction to dentin pre-treated with EDTA [17]. EDTA has also been shown to increase the survival of SCAPs by partially reversing the cytotoxicity of NaOCl [174]. EDTA's main disadvantage is its erosion of peritubular and intertubular dentin when applied for 10 minutes [101].

STEM CELLS

Without stem cells, tissue engineering and REPs would not be possible. Stem cells of the adult are one of two types, multipotent or pluripotent. This means they can either divide into another cell of the same type [multipotent) or into any type of human cell [pluripotent). It was discovered by Lovelace that the periapical area of immature teeth with necrotic pulps is an area rich in stem cells. This is why when bleeding of the apical papilla is evoked, mesenchymal stem cells flow into the root canal space [7]. These stem cells can then differentiate into one of many types of pulp cells including dental pulp stem cells (DPSC), periodontal ligament stem cells (PDLSCs), dental follicle progenitor stem cells (DFPCs), stem cells from human exfoliated deciduous teeth (SHEDs), and stem cells from the apical papilla (SCAPs) [2, 193-197]. Stem cells for endodontic regeneration are postnatal and mostly located in the cell-rich zone of the pulp, near the odontoblastic layer [198, 136]. The five types of stem cells listed above are essential for regeneration of pulp fibroblasts, extracellular matrix, and collagen [136, 195, 198]. Despite this fact, SCAP are the main source of stem cells utilized for stimulation of root development [193].

SCAFFOLD

The scaffold is the substance in which the growth factors and blood adhere to. The scaffold functions as an extracellular matrix to transport nutrients, oxygen, and metabolic waste [158]. Nevins first introduced the use of a collagen gel scaffold for REPs in 1976 [151]. There have been many authors to show the use of clotted blood as the scaffold [167]. According to Hutmacher, there are six properties of an ideal scaffold [147]. These six properties include:

1. Porous structure for tissue and vascular integration.
2. Biodegradable at a rate of tissue formation.
3. Allow cellular attachment for differentiation and proliferation.
4. The mechanical properties of the site being implanted must be adequate.
5. Does not elicit any adverse reactions.
6. Easily formed into different sizes and shapes.

Blood clots have traditionally been the scaffold material of choice. Other materials, however, have recently been investigated [147]. Several cases have been reported using platelet rich plasma (PRP) or platelet rich fibrin (PRF) as a scaffold [154, 199-201]. Many authors have found growth factors that are released by PRP and PRF [17, 89, 90].

GROWTH FACTORS

Pulp chamber reduction has been seen with the use of long-term corticosteroids in the canal system [178]. Dexamethasone has been shown to increase the differentiation of human dental pulp cells [176]. The most important thing to note is that growth factors provide the necessary mediators to facilitate tissue engineering, thereby increasing the

prognosis of REPs.

RECOMMENDED GUIDELINES FOR REPs

The AAE has published recommendations for regenerative endodontic procedures. As of 2016, these recommendations are as follows:

Case Selection:

- Tooth with necrotic pulp and an immature apex.
- Pulp space not needed for post/core, final restoration.
- Compliant patient/parent.
- Patients not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).

Informed Consent

- Two (or more) appointments.
- Use of antimicrobial(s).
- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection.
- Alternatives: MTA apexification, no treatment, extraction (when deemed nonsalvageable).
- Permission to enter information into AAE database (optional).

First Appointment

- Local anesthesia, dental dam isolation and access.
- Copious, gentle irrigation with 20 ml 1.5% NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space [e.g., needle with closed end and side-vents, or EndoVac™). Lower concentrations of

NaOCl are advised (1.5% NaOCl (20 mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues.

- Dry canals with paper points.
- Place calcium hydroxide or low concentration of triple antibiotic paste. If the triple antibiotic paste is used: 1) consider sealing pulp chamber with a dentin bonding agent [to minimize risk of staining] and 2) mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 0.1 mg/ml.
- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining).
- Seal with 3-4 mm of a temporary restorative material such as Cavit™, IRM™, glass ionomer or another temporary material. Dismiss patient for 1-4 weeks.

Second Appointment (1-4 weeks after 1st visit)

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3% mepivacaine without vasoconstrictor, dental dam isolation.
- Copious, gentle irrigation with 20 ml of 17% EDTA.
- Dry with paper points.
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with

the goal of having the entire canal filled with blood to the level of the cemento–enamel junction). An alternative to creating a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF) or autologous fibrin matrix [AFM).

- Stop bleeding at a level that allows for 3-4 mm of restorative material.
- Place a resorbable matrix such as CollaPlug™, CollaCote™, CollaTape™ or other material over the blood clot if necessary and white MTA as capping material.
- A 3–4 mm layer of glass ionomer (e.g., Fuji IILC™, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA should be considered in teeth where there is an esthetic concern.
    - Anterior and Premolar teeth - Consider use of CollaTape/CollaPlug and restoring with 3 mm of RMGI or Biodentine followed by bonding a filled composite to the beveled enamel margin.
    - Molar teeth or teeth with PFM crown - Consider use of CollaTape/CollaPlug and restoring with 3 mm of MTA, followed by RMGI or alloy.

Follow-up

- Clinical and Radiographic exam
    - No pain, soft tissue swelling or sinus tract (often observed between first and second appointments).
    - Resolution of apical radiolucency (often observed 6-12 months after treatment)
    - Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12-24 months after treatment).

- Increased root length.
- Positive pulp vitality test response

- The degree of success of regenerative endodontic procedures is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
  - Primary goal: The elimination of symptoms and the evidence of bony healing.
  - Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential)
  - Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue)

MATERIALS AND METHODS

HUMAN TEETH SELECTION

Following IRB approval, human permanent teeth were compiled and stored in 0.1-percent thymol solution at 4°C. Inclusion criteria were: roots must be completely formed, teeth without decay, and at least 4mm midroot diameter buccolingually or mesiodistally. Exclusion criteria were: hypocalcification, restorations, decay, hypoplasia, fractures or cracks, incomplete radicular formation, fluorosis, and dentinogenesis or amelogenesis imperfecta. Visual inspection was used to evaluate whether each tooth fits within these criteria.

HUMAN DENTIN SPECIMEN PREPARATION

A diamond saw with water irrigation was used to cut off the crowns of the teeth and each root was sectioned into two halves longitudinally. Dentin samples [n=126) with standard dimensions of 4x4x1.5 mm were prepared from each root section and the pulpal sides were polished. This was achieved by sequentially using 500, 1200, and 2400 grit SiC abrasive papers on a Struers Rotopol 31/Rotoforce 4 polishing unit [Struers, Cleveland, OH). The specimens were then irrigated with 1.5% NaOCl and 17% EDTA for 4 minutes to eliminate the smear layer as described in the literature [45). To avoid drying out of the specimens, continuous water irrigation was used.

COLLECTION OF BACTERIAL ISOLATES

One bacterial isolate was clinically obtained from an immature permanent tooth with pulpal necrosis that was indicated for REP [IRB #: 1510640949). This mixed

species clinical isolate was obtained while performing a regenerative endodontic procedure [under rubber dam isolation). Initiation of the endodontic access was performed with a sterile carbide bur followed by cleaning the rubber dam with 3% hydrogen peroxide and 6% sodium hypochlorite solution. A swab soaked in 6% NaOCl was then used to disinfect the pulp chamber followed by inactivation with sterile 5% sodium thiosulfate. A #15 file with the handle severed off was then used to obtain samples from the infected root canal. The canal was then instrumented 1mm short of the apical foramen for 30 seconds. The apical and radicular fluid was obtained using paper points inside the root canal system and placing them to the same working length as the canal was instrumented to for a period of 1 minute. The paper points and the #15 file were submerged into 2 mL of brain heart infusion broth containing 5 g/L of yeast extract (BHI-YE) and vortexed to elute the bacteria. These were grown in anaerobic conditions at 37ºC for 48 h and frozen in the BHI-YE culture broth supplemented with 10% glycerol at -80ºC until the bacteria were used.

BACTERIAL GROWTH ON ROOT SPECIMENS

For sterilization purposes, the radicular dentin samples (n = 42) were first exposed to ethylene oxide. Then, each specimen was placed [pulp surface facing outward) inside one well of a sterile 96 well plate. After that, 190 µL of fresh BHI-YE growth media supplemented with vitamin K/hemin and 10 µL of an overnight culture of a mixed species clinically isolated bacterial culture was added to each dentin sample and incubated for three weeks at 37°C anaerobically. Seven dentin samples were not infected [sterility control group). To achieve anaerobic conditions, the 96 well plate was placed in a BD GasPak incubation container with a BD GasPak EZ Sachet. The sachet contained a

catalyst and hydrogen gas generating system. The catalyst and hydrogen gas fuse with molecular oxygen in the gas pack container which removes the oxygen by converting it to water. This created an anaerobic environment in order for anaerobic bacteria to grow. The growth media was replaced weekly during the incubation period.

PREPARATION OF ANTIBIOTIC PASTE

Two concentrations of radiopaque DAP were prepared according to previously published studies [47, 48) with slight modifications to incorporate the radiopaque component into the pastes. In summary, 10 and 100 mg of equal portions of metronidazole and ciprofloxacin [Champs Pharmacy, San Antonio, TX) were dissolved in 10 mL of sterile water to form 1 and 10 mg/mL of DAP solutions, respectively. Thereafter, 3 g of barium sulfate [Reagent Plus, Sigma-Aldrich, St. Louis, MO) was gradually incorporated into each DAP solution under stirring to form 30% radiopaque DAP slurry composed of 30% [w/v) of barium sulfate. The 1 mg/mL radiopaque DAP had a radiodensity of 2.1 mmAl and the 10 mg/mL radiopaque DAP had a radiodensity of 1.73 mmAl. Then, 0.7 g of methylcellulose powder [Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) was gradually dissolved into each DAP slurry at room temperature to create a final pasty consistency of DAP. Finally, the radiopaque DAP was centrifuged for 15 minutes at 7000 rpm to form bubble-free homogenous injectable paste with 1 or 10 mg/mL of DAP. Furthermore, DAP-free radiopaque paste and a commercial $Ca[OH)_2$ paste [UltraCal XS, Ultradent, South Jordan, UT) was used as additional treatment groups in this study.

TREATMENT OF INFECTED DENTIN

The dentin samples were randomized into 6 experimental groups [n=7) and treated for one week with freshly made radiopaque DAP [1 and 10 mg/mL), radiopaque paste without DAP [placebo), commercial $Ca[OH)_2$ paste, no treatment control group, and a no treatment/no bacteria sterility control group. Each infected dentin sample was treated with 100 µl of the assigned paste and samples was incubated anaerobically at 37 ºC during the treatment period.

BIOFILM DISRUPTION ASSAYS

The treatment pastes were rinsed off from each dentin specimen by gently washing them with sterile saline. The dentin specimens were then transferred to a sterile plastic test tube containing 200 µL of sterile saline. To detach the bacterial biofilm, the tubes were sonicated using [NAME BRAND AND SPEED FROM MACHINE) for 20 seconds and vortexed for 30 seconds. These detached biofilm cells were diluted 1:10 and 1:1000 and spiral plated on blood agar plates [CDC, BioMerieux). The plates were incubated anaerobically for 48 h in 5% CO_2 at 37°C. An automated colony counter [Synbiosis, Inc., Frederick, MD) was utilized to enumerate the CFUs/mL.

FOLLOW-UP ANTIBACTERIAL TESTING

The same batch of radiopaque pastes were stored at 4°C for 3 and 6 months. After each time point, the antibacterial experiments of the same 6 experimental groups were repeated as described earlier in this protocol. Furthermore, the same batch of the commercial purchased calcium hydroxide paste was used at all time points.

STATISTICAL ANALYSIS

The effects of treatment and time on CFUs was evaluated using two-way ANOVA, with fixed effects for treatment, time, and the treatment-by-time interaction. Pair-wise comparisons were made using Fisher's Protected Least Significant Differences. A 5% significance level was used for all tests. The CFU data were expected to be log-normal, so a natural log transformation of the data was used in the analyses.

SAMPLE SIZE

Based on previous data, the coefficient of variation is estimated to be 0.4. With a sample size of 7 per treatment at each time point, the study had 80% power to detect a 2x difference in means between any two groups, assuming two-sided tests each conducted at a 5% significance level.

RESULTS

DIRECT ANTIBACTERIAL EFFECTS OF VARIOUS TREATMENT PASTES AFTER 0 MONTHS AGING, 3 MONTHS AGING, AND 6 MONTHS AGING

The infected dentin sample groups treated with 1 and 10mg/mL RoDAP, and calcium hydroxide exhibited a significant and statistically relevant antibacterial effect at each time point when compared to both the control groups and placebo radiopaque paste groups ($p < 0.05$; $p=0.0004$). Where T_0 = 0 months aging of pastes, T_3 = 3 months aging of pastes, T_6 = 6 months aging of pastes, the mean bacterial count values for the groups tested at each time point are as follows: Control group $T_0 = 5.95$, $T_3 = 6.09$, $T_6 = 5.47$; Placebo group $T_0 = 5.70$, $T_3 = 6.35$, $T_6 = 5.74$; 1 and 10mg/mL RoDAP groups had a mean value of 0 at all time points, and for the calcium hydroxide group $T_0 = 0$, $T_3 = 0$, $T_6 = 2.32$. This data is summarized in Table 1. There was no significant difference found between the groups tested with 1 and 10 mg/mL RoDAP and calcium hydroxide at time points T_0, T_3, or T_6. At time point T_6, however, calcium hydroxide showed less biofilm eradication than both RoDAP concentrations. This difference was not statistically significant when comparing the efficacy of either RoDAP concentration to the efficacy of calcium hydroxide at T_6 (with all p values < 0.05). However, this difference was statistically significant when comparing the antibacterial efficacy of calcium hydroxide after 3 months aging verses after 6 months of aging ($p > 0.05$).

FIGURES AND TABLES

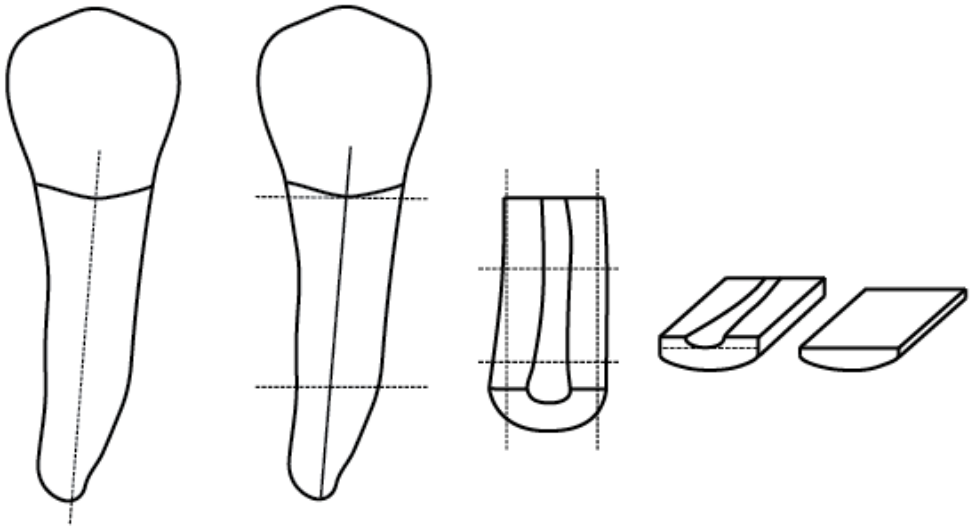


FIGURE 1. Overview of specimen preparation. Each tooth was sectioned, cut to 4x4 mm, and the inner surface was flattened.

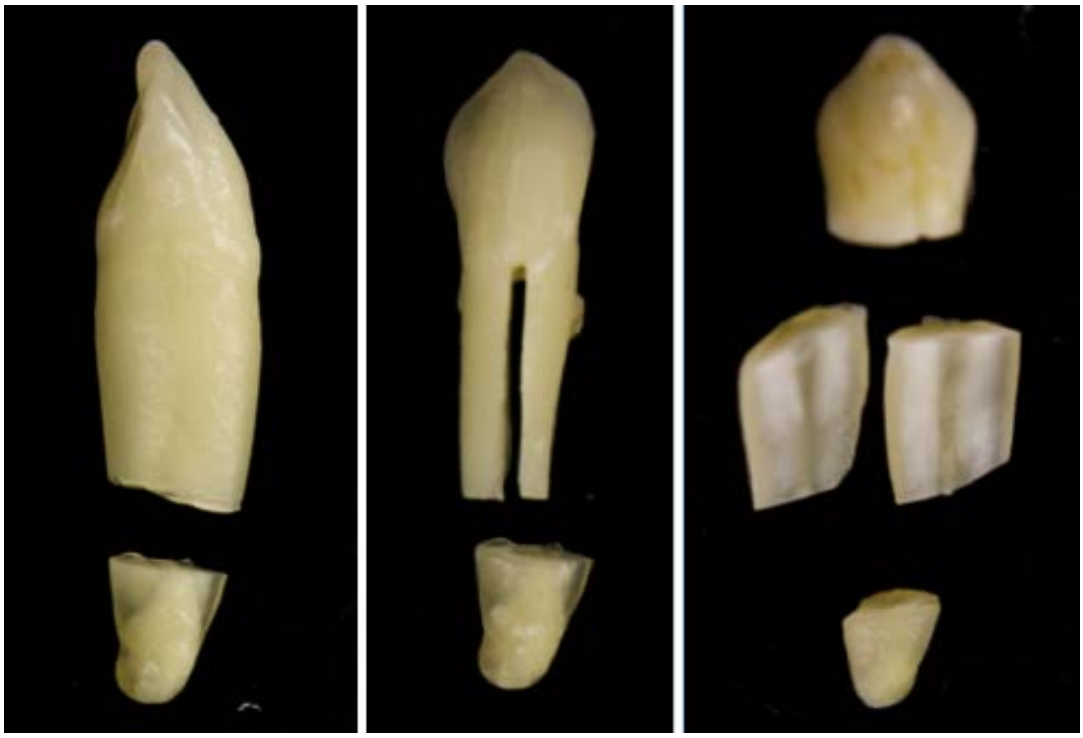
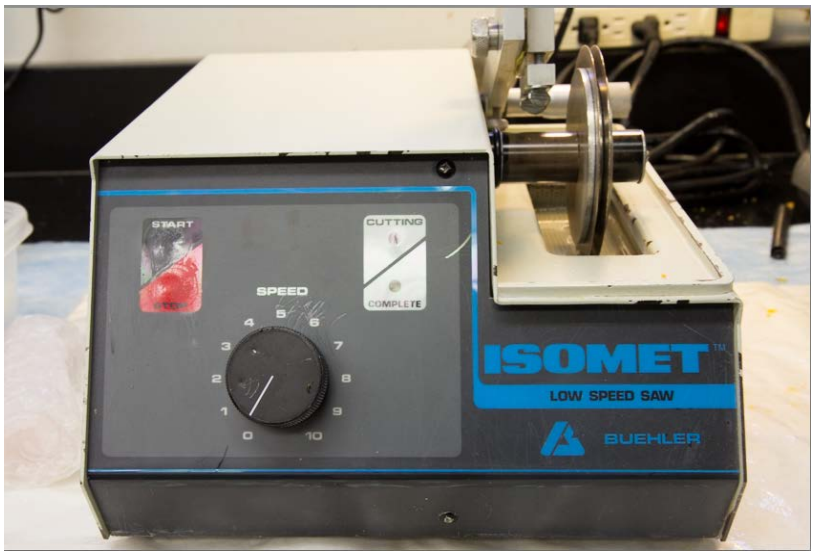


FIGURE 2. Teeth were sectioned using a high-speed saw with water irrigation.

FIGURE 3. The high-speed saw that was used with water irrigation (Lapcraft L'il Trimmer).



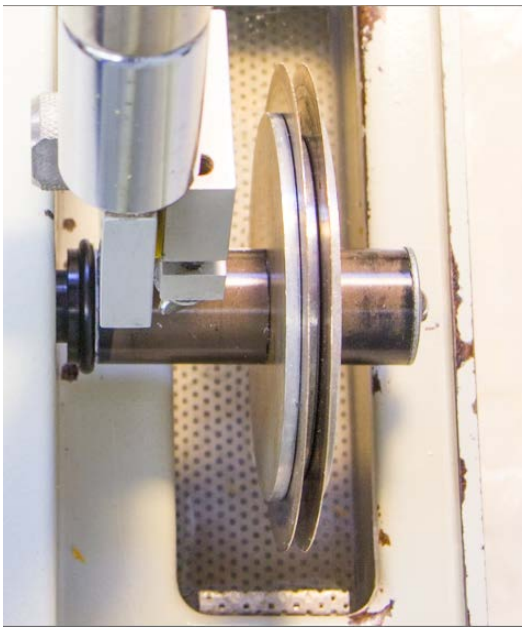


FIGURE 4. The low-speed saw used with water irrigation [Isomet, Buhler).

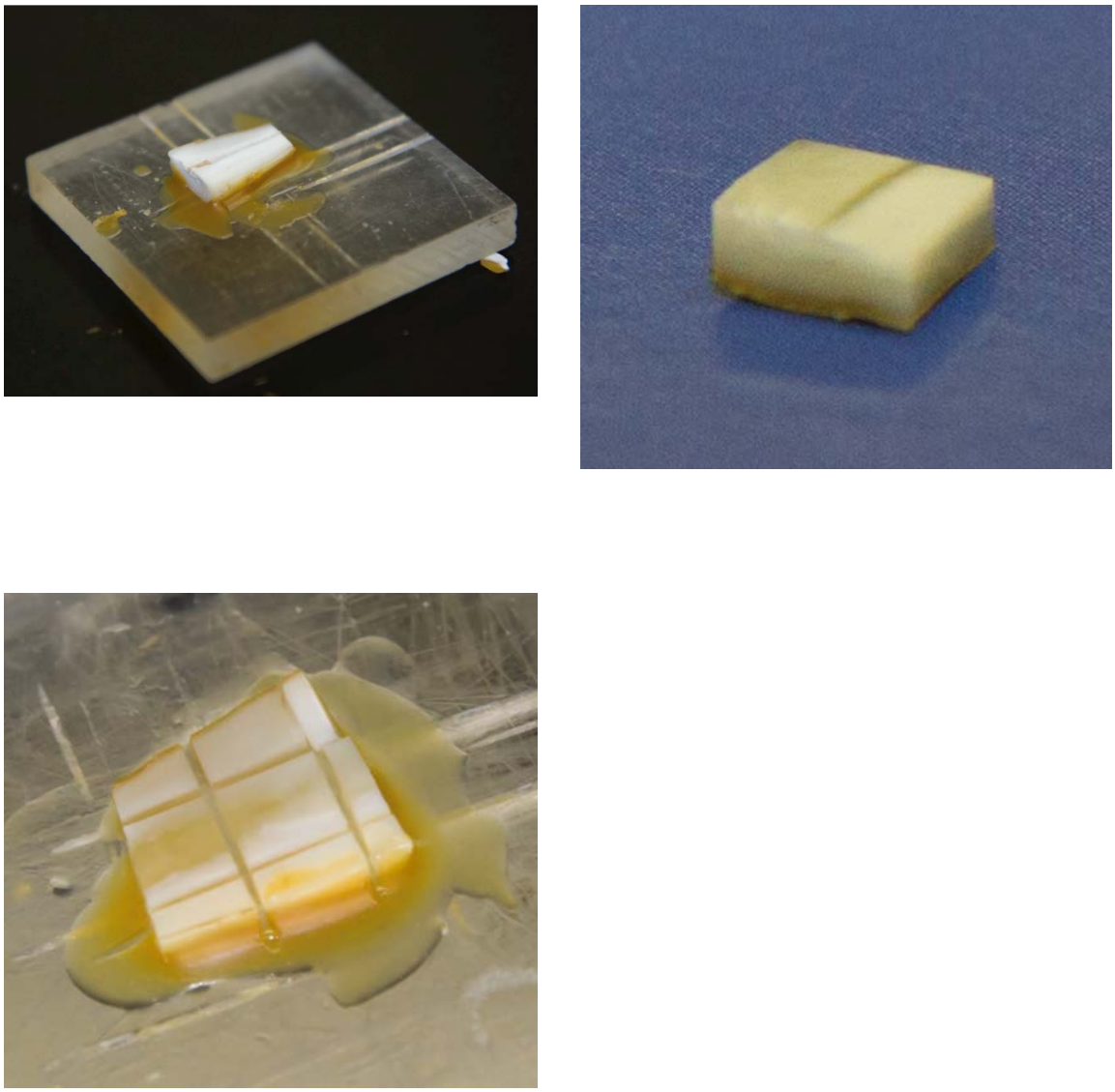


FIGURE 5. Each half-root was cut into a 4x4-mm square with a double-bladed low-speed saw with water.

FIGURE 6. Dentin specimens [4x4-mm) were mounted on Struer block for flattening and smoothing.

FIGURE 7. The RotoPol 31 [bottom) / Rotoforce-4 [top) was used to flatten the bottom side and polish the pulpal side of the specimen.

FIGURE 8. Dentin specimen after polishing, prior to sterilization.

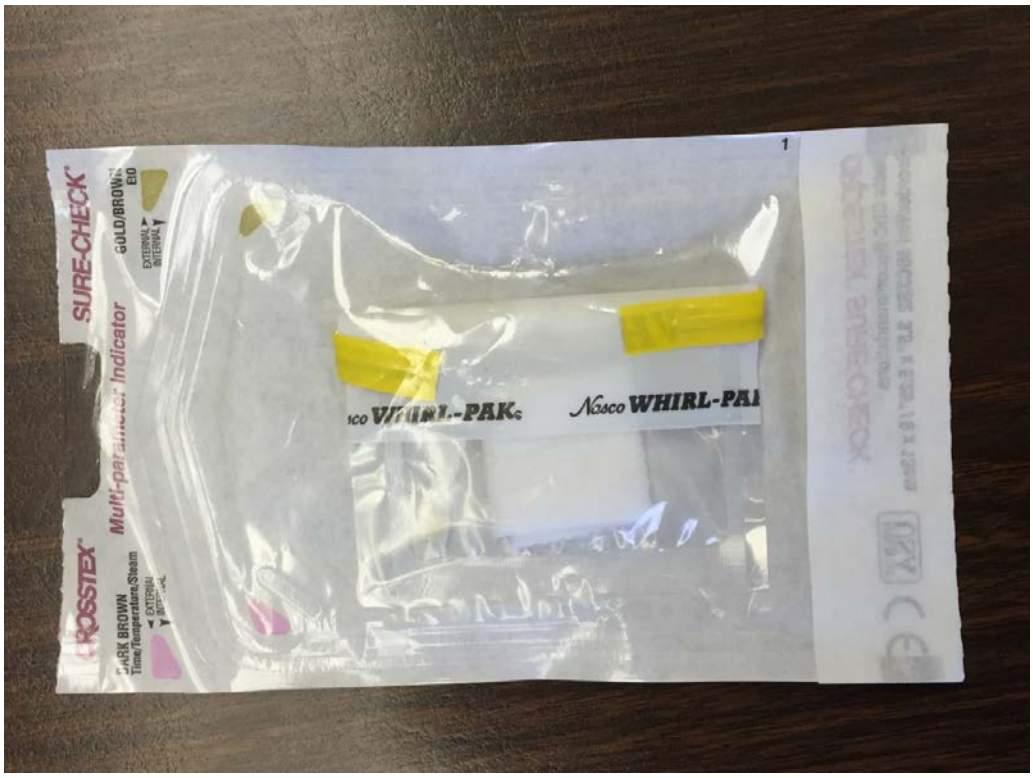


FIGURE 9 Dentin specimen were individually packaged and sterilized.

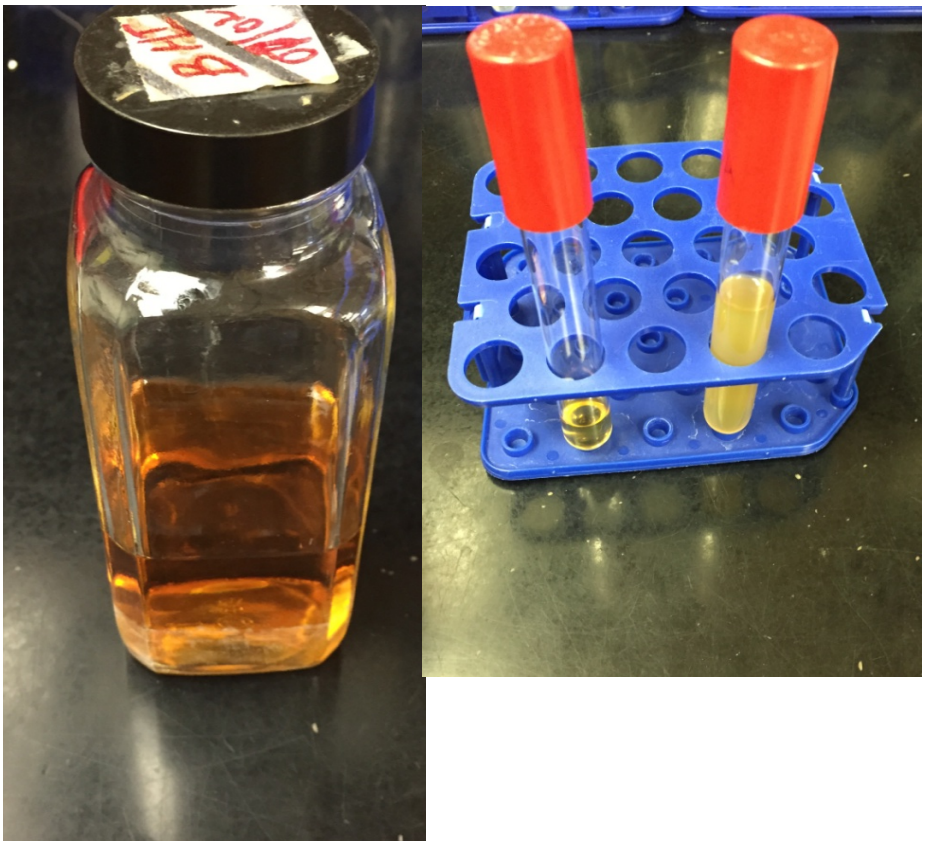


FIGURE 10. Preparation of bacteria from immature tooth with necrotic pulp with BHI media.

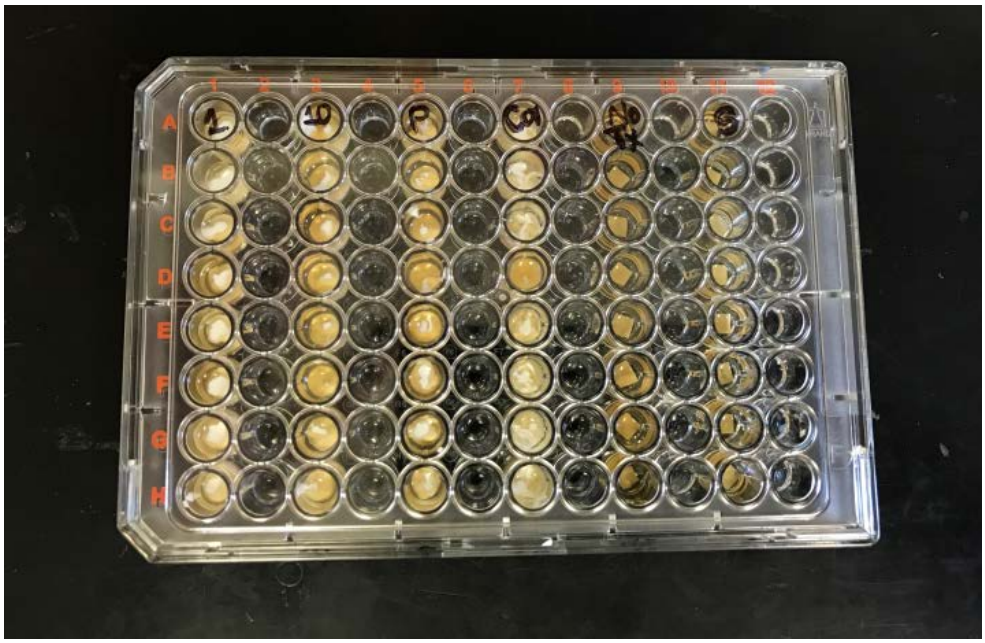


FIGURE 11. Dentin specimens with treatments placed after 3 weeks of incubation with immature biofilm. Starting from the left with group 1: 1 mg/mL RoDAP; group 2: 10 mg/mL RoDAP; group 3: barium sulfate methycellulose with no DAP; group 4: calcium hydroxide; group 5: no treatment. Group 6: sterile control.

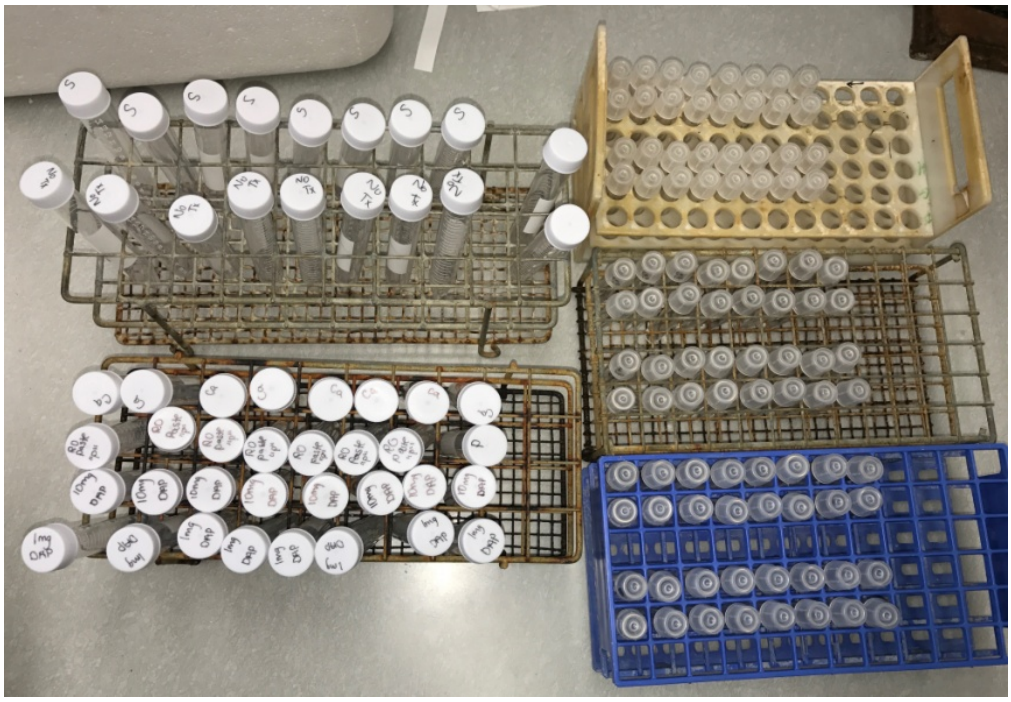


FIGURE 12. Dilutions of treatment groups after being treated before sonication and spiral plating

FIGURE 13. Blood agar plates after spiral plating and before being placed in the incubator.

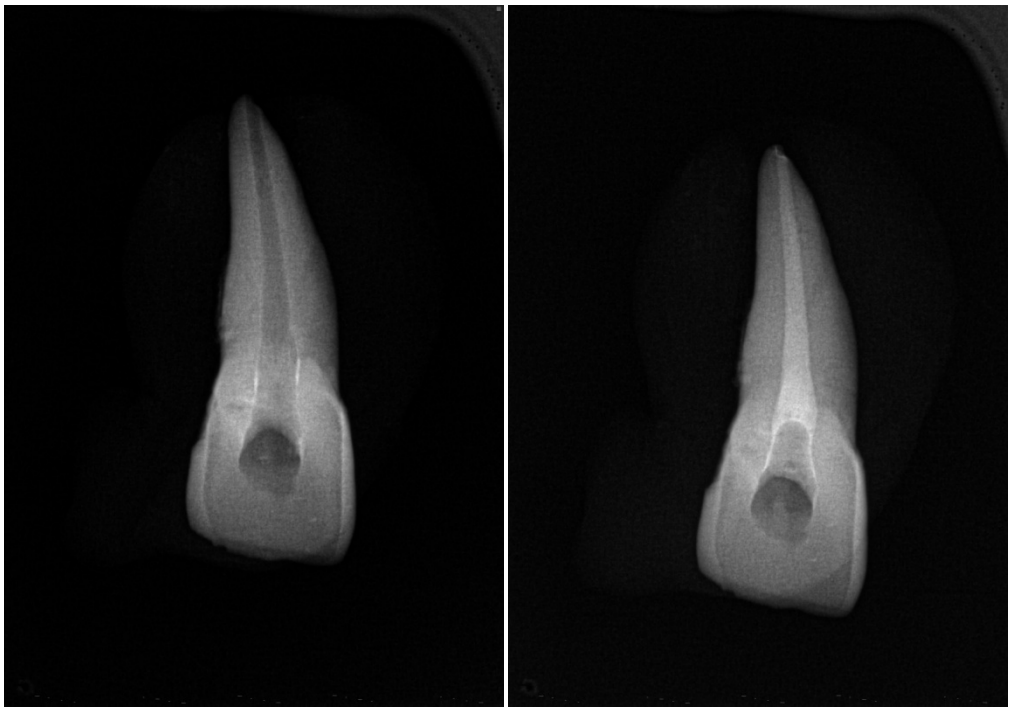


FIGURE 14. Radiograph of tooth filled with original DAP (left) and radiograph of tooth filled with RoDAP (right).

TABLE I

The mean and SE of log CFL/mL for bacterial biofilm grown for 3 weeks on dentin then treated at 0, 3, or 6 months of aging with different concentrations of RoDAP, placebo, $Ca(OH)_2$, no treatment, or sterile

Time	Treatment	N	Mean	SD	SE	Median	Q1	Q3	Min	Max
0	1 mg/mL	8	0	0	0	0	0	0	0	0
0	10 mg/mL	8	0	0	0	0	0	0	0	0
0	Placebo	8	5.70	0.15	0.05	5.66	5.60	5.83	5.52	5.92
0	CaOH	8	0	0	0	0	0	0	0	0
0	No Treatment	8	5.95	0.02	0.01	5.96	5.94	5.96	5.92	5.97
0	Sterile	8	0	0	0	0	0	0	0	0
3	1 mg/mL	8	0	0	0	0	0	0	0	0
3	10 mg/mL	8	0	0	0	0	0	0	0	0
3	Placebo	8	6.35	0.10	0.03	6.34	6.30	6.41	6.19	6.52
3	CaOH	8	0	0	0	0	0	0	0	0
3	No Treatment	8	6.09	0.16	0.06	6.11	5.97	6.21	5.85	6.27
3	Sterile	8	0	0	0	0	0	0	0	0
6	1 mg/mL	8	0	0	0	0	0	0	0	0
6	10 mg/mL	8	0	0	0	0	0	0	0	0
6	Placebo	8	5.74	0.32	0.11	5.81	5.68	5.90	5.02	6.10
6	CaOH	8	2.32	1.96	0.69	3.31	0	3.81	0	4.31
6	No Treatment	8	5.47	0.25	0.09	5.49	5.35	5.61	5.01	5.86
6	Sterile	8	0	0	0	0	0	0	0	0

TABLE II

Statistical comparison of the effect of treatment at different time points using Wilcoxon rank-sum tests

Treatment 1	**Treatment 2**	**Change from Time 0 to 3** **p-value**	**Change from Time 0 to 6** **p-value**	**Change from Time 3 to 6** **p-value**
1 mg/mL	10 mg/Ml	1.0	1.0	1.0
1 mg/mL	Placebo	0.0004	0.4002	0.0004
1 mg/mL	CaOH	1.0	0.0126	0.0126
1 mg/mL	No Treatment	0.0821	0.0004	0.0004
1 mg/mL	Sterile	1.0	1.0	1.0
10 mg/mL	Placebo	0.0004	0.4002	0.0004
10 mg/mL	CaOH	1.0	0.0126	0.0126
10 mg/mL	No Treatment	0.0821	0.0004	0.0004
10 mg/mL	Sterile	1.0	1.0	1.0
Placebo	CaOH	0.0004	0.0818	0.0009
Placebo	No Treatment	0.0009	0.0181	0.8748
Placebo	Sterile	0.0004	0.4002	0.0004
CaOH	No Treatment	0.0821	0.0009	0.0009
CaOH	Sterile	1.0	0.0126	0.0126
No Treatment	Sterile	0.0821	0.0004	0.0004

TABLE III

Statistical comparison of the effect of treatment over time using Wilcoxon rank-sum tests

Treatment 1	Treatment 2	Change from Time 0 to 3 p-value	Change from Time 0 to 6 p-value	Change from Time 3 to 6 p-value
1 mg/mL	10 mg/mL	1.0	1.0	1.0
1 mg/mL	Placebo	0.0004	0.4002	0.0004
1 mg/mL	CaOH	1.0	0.0126	0.0126
1 mg/mL	No Treatment	0.0821	0.0004	0.0004
1 mg/mL	Sterile	1.0	1.0	1.0
10 mg/mL	Placebo	0.0004	0.4002	0.0004
10 mg/mL	CaOH	1.0	0.0126	0.0126
10 mg/mL	No Treatment	0.0821	0.0004	0.0004
10 mg/mL	Sterile	1.0	1.0	1.0
Placebo	CaOH	0.0004	0.0818	0.0009
Placebo	No Treatment	0.0009	0.0181	0.8748
Placebo	Sterile	0.0004	0.4002	0.0004
CaOH	No Treatment	0.0821	0.0009	0.0009
CaOH	Sterile	1.0	0.0126	0.0126
No Treatment	Sterile	0.0821	0.0004	0.0004

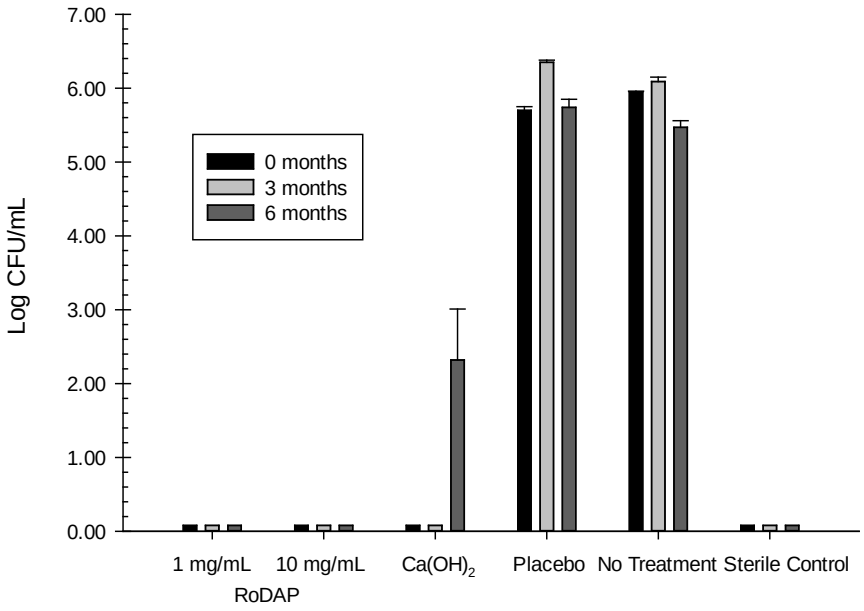
TABLE IV

Statistical comparison of the effect of time, by treatment using Wilcoxon signed rank tests

Treatment	**Times**	**p-value**
1 mg/mL	0 vs. 3	Cannot calculate (zero at both times)
10 mg/mL	0 vs. 3	Cannot calculate (zero at both times)
Placebo	0 vs. 3	0.0078
CaOH	0 vs. 3	Cannot calculate (zero at both times)
No Treatment	0 vs. 3	0.0391
Sterile	0 vs. 3	Cannot calculate (zero at both times)
1 mg/mL	0 vs. 6	Cannot calculate (zero at both times)
10 mg/mL	0 vs. 6	Cannot calculate (zero at both times)
Placebo	0 vs. 6	0.8438
CaOH	0 vs. 6	0.0625
No Treatment	0 vs. 6	0.0078
Sterile	0 vs. 6	Cannot calculate (zero at both times)
1 mg/mL	0 vs. 6	Cannot calculate (zero at both times)
10 mg/mL	3 vs. 6	Cannot calculate (zero at both times)
Placebo	3 vs. 6	0.0078
CaOH	3 vs. 6	0.0625
No Treatment	3 vs. 6	0.0078
Sterile	3 vs. 6	Cannot calculate (zero at both times)

TABLE V

Antimicrobial effect of RoDAP and $Ca(OH)_2$ at 0, 3, and 6 months on a mixed species biofilm from an immature tooth with necrotic pulp



DISCUSSION

Immature teeth with necrotic pulps present with a unique challenge to the dental practitioner due to the thin, weak dentinal walls, short roots, and open apices. Traditional treatments for these teeth include apexification with calcium hydroxide or MTA, with the goal being to form an apical barrier so that root canal treatment can be completed. The problem with these treatments is that it leaves the immature tooth with thin, weak dentinal walls and short root length. Regenerative endodontic procedures are becoming more popular in the treatment of these immature teeth with necrotic pulps. The AAE recommends performing a regenerative endodontic procedure when the patient presents with a tooth with a necrotic pulp and immature apex, the pulp space is not needed for a post, core or final restoration, the patient/parent is compliant, and the patient is not allergic to medicaments and antibiotics necessary to complete the procedure.

Three things are necessary to perform a regenerative endodontic procedure: stem cells, a scaffold, and growth factors. These things cannot be effective in tissue engineering, however, without adequate disinfection of the root canal system [16]. This is the area of focus in this study. Specifically, we focused on the disinfection of the canal system using a novel radiopaque double antibiotic paste (DAP). DAP has emerged in the literature as of late due to its promising antibacterial properties and the minimal tooth discoloration it causes when compared to TAP [42, 201]. In 2017, an in vitro study by Jacobs et al. showed that DAP had a significant direct antibacterial effect on both mature and immature biofilms. Additionally, they found that 5mg/mL of DAP exhibited a significant residual antibacterial effect against biofilm from an immature tooth, whereas

calcium hydroxide did not show any residual antibacterial effects [202]. This is in agreement with recent studies performed by Jenks et al, where they also did not find any residual antibacterial effect with calcium hydroxide[203].

In this study, we aimed to test a novel radiopaque double antibiotic paste (RoDAP). The purpose was to test its direct antibacterial effect as well as the shelf life of this new paste. The significant difference with this new RoDAP in comparison to original DAP is the addition of barium sulfate. The barium sulfate was added to serve as a radiopacifier. Unlike commercially available calcium hydroxide pastes, all traditional antibiotic mixtures used during REPs lack radiopacity and therefore cannot be visualized on radiographs. Furthermore, all commercially available antibiotic intracanal medicaments, specifically Odontpaste and Ledermix, are not radiopaque. This limits the use of these antibiotic medicaments in the United States because they are not helpful in detecting lateral and accessory root canals, areas of root resorption or fractures, and the degree of apical development. The incorporation of radiopaque agents into antibiotic medicaments may offer an additional diagnostic aspect to these pastes as well as help the clinicians to recognize the extrusion of antibiotic paste beyond the root canal system. The accuracy of its placement is of great important during REPs due to the presence of large blunder buss apices of immature teeth with necrotic pulps.

In this study, we wanted to find out if the addition of these insoluble radiopaque agents may interfere with the antibacterial abilities of the antibiotics. A recent study at Indiana University School of Dentistry introduced a radiopaque DAP with comparable radiodensity to the commercially available calcium hydroxide paste. The proposed radiopaque DAP was composed of 30% insoluble barium sulfate as a contrasting agent.

This is why we chose to use 30% barium sulfate in our RoDAP for this study. To the best of our knowledge, the antibacterial stability of this novel RoDAP had not been tested prior to this study.

To test the new RoDAP, we infected sterile dentin specimens with biofilm from an immature tooth with a necrotic pulp. We then tested the direct antibacterial efficacy of 1mg/mL RoDAP, 10 mg/mL RoDAP, $Ca(OH)_2$, and placebo paste (methycellulose only) on the infected dentin specimens at 0, 3, and 6 months of shelf life. We found both concentrations of RoDAP to have substantial antimicrobial effects at all time points. Calcium hydroxide also showed substantial antimicrobial effects at all time points. At the 6 month time point, however, calcium hydroxide showed slightly less antimicrobial activity and this difference was statistically significant.

One of the limitations of this study was the use of a biofilm from a single immature tooth. We recommend that further studies be done to test the antibacterial efficacy and shelf life of RoDAP against multispecies biofilms from multiple teeth, mature and immature. Additionally, there have been no studies to test RoDAP and the effect of barium sulfate on the survivability of stem cells of the apical papilla (SCAP). However, studies such as these have been performed with other materials containing barium sulfate, such as calcium hydroxide. It would be wise to see further studies on how different concentrations of RoDAP affect the survival of SCAP and how this compares to DAP without barium sulfate.

In conclusion, RoDAP may be a promising new material to be available commercially for regenerative endodontic procedures. With a shelf life of up to six months, significant radiopacity, and a strong direct and indirect antimicrobial effect, it

may become the modern clinicians' first choice of an intracanal medicament for regenerative endodontic procedures.

SUMMARY AND CONCLUSIONS

Treatment of infected dentin with 1 mg/mL and 10 mg/mL of RoDAP, and $Ca(OH)_2$ demonstrated significant and substantial antibiofilm effects in comparison with untreated control groups or groups treated with placebo paste after 0 months, 3 months, and 6 months of treatment paste aging. Calcium hydroxide, however, showed slightly less antibiofilm activity after 6 months of aging when compared with 0 months and 3 months of aging. This difference was statistically significant ($p > 0.05$). In conclusion, both concentrations of RoDAP maintain full antibacterial efficacy after 6 months of aging while calcium hydroxide lost some antibacterial activity after a shelf life of 6 months.

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ABSTRACT

THE ANTIBACTERIAL STABILITY OF A NEW RADIOPAQUE DOUBLE ANTIBIOTIC PASTE

by

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We evaluated the antibacterial stability (shelf life) of a new radiopaque double antibiotic paste (RoDAP) loaded in a methylcellulose system with 30% w/v barium sulfate against biofilm collected from an immature tooth with necrotic pulp. Uniform radicular dentin specimens were infected with bacterial biofilm obtained from an immature tooth with a necrotic pulp and incubated anaerobically for three weeks. These samples were randomly divided into 6 experimental groups (n=7) and treated for 1 week at three time points of aged radiopaque DAP: 0 months, 3 months, and 6 months. Group 1: 1mg/mL RoDAP treatment. Group 2: 10 mg/mL RoDAP treatment. Group 3: Calcium hydroxide ($Ca(OH)_{2)}$ treatment. Group 4: Methylcellulose with barium sulfate. Group 5: No treatment. Group 6: No bacteria or treatment. The samples were rinsed with sterile saline to detach biofilms and then spiral plated using a biofilm disruption assay. Statistical analyses were performed using Wilcoxon rank-sum tests and Wilcoxon signed

rank tests with fixed effects for treatment, time, and the treatment-by-time interaction. Treatment of infected dentin with 1 mg/mL RoDAP, 10 mg/mL RoDAP, and $Ca(OH)_2$ demonstrated significant and substantial antibiofilm effects in comparison to untreated control groups or groups treated with placebo paste after 0, 3, and 6 months of aging. Calcium hydroxide, however, showed slightly less antibiofilm activity after 6 months of aging when compared to 0 months and 3 months of aging. This difference was statistically significant ($p > 0.05$). In conclusion, both concentrations of RoDAP maintained full antibacterial efficacy after 6 months of aging, while calcium hydroxide lost some antibacterial activity after a shelf life of 6 months.

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